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14. **ABSTRACT**

The sentinel lymph node (SLN) is the first node in the mammary gland to harbor malignant cells in breast tumors with metastasis, and SLN positivity is an indication for axillary lymph node dissection. The purpose of our study is to identify specific genetic alterations using array-CGH in the metastatic sentinel lymph node lesions, in comparison to the ones observed in the corresponding primary tumors from patients with breast cancer. We believe that the characterization of genetic alterations at the SLN site is a logical step to define the cytogenetic evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur in the early metastatic process, before distant metastasis occur. Ultimately these alterations can be used as molecular markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary dissection, in the management of breast cancer patients.

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Detection of genetic alterations in breast sentinel lymph node by array-CGH
P.I. Luciane R.Cavalli, PhD

Introduction

In breast cancer, axillary lymph node status remains the single most important prognostic variable and a crucial component to the staging system. The sentinel lymph node (SLN) is the first node to harbor malignant cells in breast tumors with metastasis, and SLN positivity is an indication for axillary lymph node dissection (Veronesi et al, 2001). Therefore the accurate evaluation of the SLN, to identify even the smallest metastatic foci at this site is essential prior to complete axillary lymph node dissection. Progress in this evaluation would include the development of better methods to predict prognosis on the basis of molecular markers. Several markers, such as *ERBB-2*, *TP53, VEGF*, have been shown to have significant prognostic potential in breast cancer with respect to the axillary and systemic metastasis (Linderholm et al. 1998; Thor et al. 1998; Gillanders et al. 2004). However, none of these markers can be used either separately or in combination to influence the decision for an axillary node dissection once tumor cells are detected in the SLN (Lindahl et al, 2000; Hery et al, 2002; Guillanders et al, 2004).

Recently there have been several published reports aiming at "profiling" breast tumors and predicting their metastasis (Perou et al, 2000; Sorlie et 2001; West et al, 2001; Van de Vijver et al, 2002; Van't Veer et al, 2003; Weigelt et al, 2003). However none of them have been based on the SLN status, but rather on more distant axillary metastasis. In addition, very few of these studies have been performed in paired primary tumors and metastatic lesions from the same patient. We previously reported the only study where genetic and epigenetic alterations were evaluated in SLN metastasis in comparison to primary tumors from the same patient (Cavalli et al, 2003). In our study, although a small number of cases was investigated using Comparative Genomic Hybridization (CGH) and methylation assays, we observed that, in general, in each separate case the number and complexity of the alterations found between the two groups were similar, and in every case there were at least two chromosomal abnormalities common to both the primary tumor and the SLN metastasis groups. No significant difference in the total number of chromosomal changes was observed between these two groups.

CGH is a highly informative methodology for the identification of copy number changes in tumors, however it is restricted to a level of resolution of about 5-10Mb (Kallioniemi et al, 1992). The newly developed array-CGH, has been used for high-resolution mapping of gains and losses of the human genome in a single experiment, allowing a locus-by-locus DNA copy number evaluation (Pinkel et al, 1998). Recent studies have compared data obtained from conventional CGH analysis (i.e. performed on normal chromosomes) to array-CGH data from the same tumors and found a high rate of concordance between the two methods. For instance, Yano *et al* (2004) found over 94% of concordance with respect to loss of genetic material in samples of prostate cancer, when analyzed by both conventional CGH and array-CGH. These authors concluded that in general, the chromosomal aberrations detected by array-CGH were in good agreement with those by conventional CGH.

In this study we will investigate the chromosomal aberrations in breast sentinel lymph nodes using CGH. The assessment of copy number changes in primary breast tumors and their corresponding metastatic lesions, can define whether metastatic lesions exhibit the same type of

genetic alterations found in primary tumors, or whether there are some types of alterations in primary tumors that make metastases more likely to occur.

We believe that the characterization of genetic alterations at the SLN metastasis is a logical step in defining the evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur early in the metastatic process, before distant metastasis takes place. The identification of this subset of genes is critical in the progression of the primary tumor to an early metastatic disease, and will allow us to build a classifier to predict breast cancer metastasis, identifying individuals at different risks of developing axillary lymph node metastasis.

Body

The main purpose of this study is to identify specific genetic alterations in the metastatic sentinel lymph node lesions, in comparison to the ones observed in the corresponding primary tumors from patients with breast cancer using Comparative Genomic Hybridization (CGH).

In this first year of the award, we have recruited a graduate student, Ms. Savana Santos, to work on this project. We have investigated the DNA copy number alterations, using the CGH method, a well established methodology in our laboratory at Georgetown University, in a total of 14 paraffin-embedded samples from patients with breast cancer. Alterations in the DNA copy number have been found in all the samples analyzed. The most frequent gains and loss observed were: -13q13-q32, +6q13-q23, +11p15-q21,+12q23-ter +16, +20. Loss on 6q and gain of chromosome 20 were more frequently observed in the SLN group, whereas gain on 12q and 20q were more frequent seen in the primary tumors. Gain on 6p, observed in 33.3% of the cases was only observed in the SLN group. The 13q loss, the most common abnormality in this study, was equally observed in both groups. A representative profile of a primary tumor and sentinel lymph node from the same case is shown in Figure1 (appendice).

Our final goal is to analyze a total of 30 paired samples of primary tumor and sentinel lymph node metastatic lesions from the same patient. Although we have collected most of these samples, we had technical difficulties in the CGH analysis of the initial samples of sentinel lymph node metastatic lesions (which are usually very small lesions). The DNA obtained from these samples, after celular microdissection, were not of adequate quantity and quality for the labeling process required for CGH. This technical problem has now been solved with the use of a DNA labeling method specific for microdissected samples.

Our initial analysis of the 14 breast cancer cases was performed using conventional CGH. We have initiated an active collaboration with Dr. Thomas Ried's group at the National Institute of Cancer (NCI) at NIH, and will conduct a parallel analysis of the same tumors using array CGH, which is well established in his lab. There are several advantages for testing the samples by conventional CGH prior to array-CGH analysis, including the fact that this will allow us to select the samples with "good" quality DNA to test with arrays (to save on the cost of using arrays for poor DNA quality samples which are likely to fail) and also will allow us to compare the data generated by these two methods.

In this additional year of the grant we will complete the CGH analysis of the samples collected using both CGH methods. A comparison of the genetic alterations observed between these two methodologies will be performed.

Key research accomplishments

- Accrual of 20 samples of paraffin embedded primary breast tumors and their corresponding sentinel lymph nodes metastatic lesions
- Pathology review of all the samples collected
- Cellular microdissection and DNA isolation of 14 of the samples collected
- CGH analysis of 14 samples of primary tumors and corresponding SLN metastatic lesions
- Optimization of a protocol of DNA labeling for the array-CGH experiments and establishment of a research collaboration with Dr.Thomas Ried's laboratory.

Reportable outcomes

Two abstracts presented at scientific meetings (see appendices)

1. "Detection of genetic alterations in breast sentinel lymph nodes by CGH."
Presented at the Era of Hope Meeting- Philadelphia, PA, June 8-11, 2005

2. "DNA copy number changes in breast sentinel lymph node metastasis."
To be presented at the AACR - Frontiers in Cancer Prevention Research Meeting – Baltimore, MD, October 30th - November 2nd, 2005

Conclusions

In this first year of the grant, we have established the groundwork for the success of this project. We completed the CGH analysis of a total of 14 cases of primary breast tumors and their corresponding SLN. Analysis of additional number of cases is in progress in order to confirm our preliminary results and to correlate specific genetic changes to each group of lesions studied. Currently we are performing CGH in additional paired samples to complete the analysis of a total of 30 cases. Our next step is to analyze these same samples using array-CGH, a higher resolution method for detection of genetic alterations.

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Appendices

Two abstracts presented at scientific meetings
Figure 1

1. "Detection of genetic alterations in breast sentinel lymph nodes by CGH."
LR Cavalli, SL Santos, CA Urban, Lima RS, BR Haddad
Presented at the Era of Hope Meeting- Philadelphia, PA, June 8-11, 2005

Background: Genome-wide based methodologies can reveal genes or chromosomal regions specifically altered during the process of breast cancer progression. Genetic studies comparing changes in distant metastatic lesions with those found in the corresponding primary tumors have revealed different alterations between these lesions, suggesting that specific events may be associated with metastatic dissemination. The genetic analysis of paired samples has made it possible to assess the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. In breast cancer, axillary lymph node status remains the single most important prognostic variable and a crucial component to the staging system. The sentinel lymph node (SN) is the first node to harbor malignant cells in breast tumors with metastases, and SN positivity is an indication for axillary lymph node dissection.
Purpose/Rationale: The purpose of this study is to identify specific genetic alterations in the metastatic sentinel lymph node lesions, in comparison to the ones observed in the corresponding primary tumors from patients with breast cancer using Comparative Genomic Hybridization (CGH). The characterization of genetic alterations at the SN site is a logical step to define the cytogenetic evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur in the early metastatic process.
Methods: The tissue samples are obtained from paraffin embedded archival blocks. Prior to the CGH analysis, after a histological evaluation, sections of the primary and the metastatic tumor tissue are microdissected using a modified razor blade to ensure minimal contamination of normal or stromal cells. The DNA is extracted from these sections (average 5 slides with 5mM sections per sample) and labeled by nick-translation. CGH followed previously described protocol.
Results: Eight pairs of primary tumors and SN metastatic lesions were analyzed by conventional CGH. Chromosomal abnormalities were observed in all the cases. The most frequent gains and loss observed were: -13q13-q32, +6q13-q23, +11p15-q21,+12q23-ter +16, +20. Loss on 6q and gain of chromosome 20 were more frequently observed in the SLN group, whereas gain on 12q and 20q were more frequent seen in the primary tumors. Gain on 6p, observed in 33.3% of the cases was only observed in the SN group. The 13q loss, the most common abnormality in this study, was equally observed in both groups.
Future: We plan to study total number of 30 paired samples using CGH.
Relevance: The identification of genetic alterations present in the SN of the breast will be important to detect the early genetic alterations that occur in the metastatic process. Ultimately these alterations can be used as additional molecular markers, that can help in the reduction or elimination of the need for invasive surgical procedures.
Acknowlegments: "The US Army Medical Research and Materiel Command under W81XWH-04-1-0671 supported this work.

2. "DNA copy number changes in breast sentinel lymph node metastasis."
LR Cavalli, SL Santos, EM Ribeiro, CA Urban, RS Lima, IJ Cavalli, BR Haddad.
Presented at the AACR - Frontiers in Cancer Prevention Research Meeting – Baltimore, MD, October 30th - November 2nd, 2005

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DNA copy number changes in breast sentinel lymph node metastasis.

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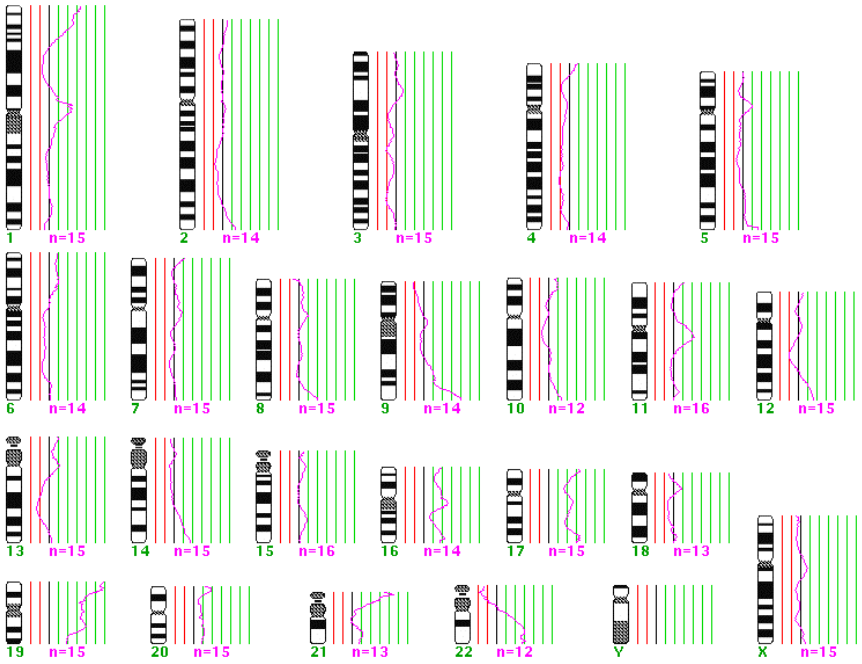
The sentinel lymph node (SLN) is the first node in the axilla to harbor malignant cells in breast tumors with metastasis, and its positivity is an indication for axillary lymph node dissection. Characterization of genetic alterations in the SLN metastatic lesions is a logical step for better defining the evolution of primary tumors to a metastatic state, and may represent the initial genetic events that occur early in the metastatic process, before distant metastasis takes place. Several studies have been performed to "profile" breast tumors and their metastatic lesions in the distant axillary lymph nodes, but none has looked at metastasis in SLN. In addition, very few of these studies have been performed in paired primary tumors and metastatic lesions from the same patient. Here we describe the results of DNA copy number changes observed in paired primary tumors and their corresponding metastatic sentinel lymph node lesions using Comparative Genomic Hybridization (CGH) analysis. The tissue blocks were obtained from the Hospital Nossa Senhora das Gracas, Brazil. CGH was performed using tumor DNA obtained from malignant cells isolated by microdissection from paraffin embedded archival material. A total of ten pairs of primary tumors and SLN metastatic lesions were analyzed. Chromosomal abnormalities were observed in all the cases. The most frequent gains (+) and losses (-) observed were: -1p31~p21, +17, +19 and +20. Theses alterations were observed in both groups. Gain on chromosome 20 was the most frequently observed in the primary tumor group, whereas losses on 1p31~p21 and gains on chromosomes 17 and 19 were equally observed in both groups. An additional number of paired samples of primary tumors and corresponding SLN metastatic lesions are currently being evaluated. This study will allow the assessment of the degree of clonal divergence and genetic heterogeneity that characterize the metastatic process. Identification of genetic alterations present in the SLN of the breast will be important to detect early genetic alterations that occur in the metastatic process. Ultimately these alterations can potentially be used as additional molecular markers that can help in the reduction or elimination of the need for invasive surgical procedures, such as axillary lymph node dissection.

Figure 1. Representative profiles of a primary breast tumor (A) and its corresponding sentinel lymph node metastatic lesion (B), showing chromosomal gains and losses. The vertical lines on the right side of the chromosome ideograms reflect different values of the fluorescence ratio between the test and the normal DNA. The values are 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 from left to right. Ratios of 1.25 or higher reflect gains whereas rations of 0.75 or lower reflect losses. N is the number of chromosomes used to generate each ratio profile.

(A)



(B)